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A RAPID METHOD FOR DETERMINING FREE AND ESTERIFIED CHOLESTEROL IN PLASMA EXTRACTS

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Running Head: Free and Esterified Cholesterol in Plasma Extracts

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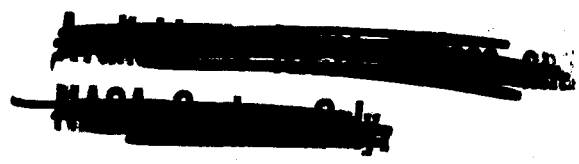
SUMMARY A method has been developed for the quantitative measurement of free and esterified cholesterol in plasma extracts. A fluorometric method was used for determining these components which were separated by thin-layer chromatography.

Free and esterified cholesterol have been determined after separation by column chromatography (1) or digitonin precipitation (2) utilizing the Liebermann-Burchard color reaction (3,4). Albers and Lowery (5) developed a fluorometric method sensitive to 0.1 μg for determining cholesterol. The following method utilizes the speed and high resolution of thin-layer chromatography for separation and the sensitivity of fluorometry for quantitation. As little as 0.2 ml of plasma or serum may be used in the determinations.



MATERIALS AND METHODS

All chemicals were reagent grade. Diethyl ether, n-hexane, and chloroform were redistilled over $FeSO_4$ and stored under nitrogen. Anhydrous methanol (Baker) was used without further purification. Cholesterol (California Corporation for Biochemical Research, Los Angeles, California) was twice recrystallized with absolute ethanol and dried to constant weight under vacuum. Cholesterol palmitate, > 99% (Applied Science Laboratories, State College, Pennsylvania), was used without further purification.

2,7,-dichlorofluorescein reagent: 400 mg 2,7,-dichlorofluorescein (Eastman) per liter in 50% ethanol.

EXTRACTION

Plasma samples were extracted with 2:1 chloroform-methanol (v/v) as described by Albrink (6). A minimum of 0.2 ml of plasma extracted with 4.0 ml of chloroform-methanol was sufficient for determining total cholesterol and the ratio of free to esterified cholesterol. Other lipid constituents may be determined on the same extract if the volume of plasma and solvent is increased. The results presented in this paper were obtained on 1.0 ml plasma samples extracted with 20.0 ml chloroform-methanol.

The samples were extracted for 1 hr in 50 ml round-bottom centrifuge tubes. The tubes were agitated with a gentle horizontal motion on a shaking machine. Twenty ml of distilled water were then added down the side of the tubes. The tubes were capped and allowed to stand overnight in the refrigerator.

The extraction mixture separated into a lower chloroform phase and an upper aqueous phase. The precipitated proteins concentrated as a disc between the two clear phases. A pipet attached to a syringe was carefully passed down the side of the tube through the aqueous phase and protein disc to remove the chloroform phase. A 0.2 ml aliquot of the extract was taken for total cholesterol assay; 1.0 ml was used for determining free and esterified cholesterol. A reagent blank containing 21.0 ml distilled water and 20.0 ml chloroform-methanol was carried along with the plasma samples.

A standard mixture of free and esterified cholesterol was prepared to approximate rat plasma concentrations. Aliquots containing 30-45 μg of cholesterol were used for both direct total cholesterol determinations and for thin-layer chromatography of free and esterified cholesterol.

THIN-LAYER CHROMATOGRAPHY

Thin-layer plates (20 cm × 20 cm) were coated with a layer of Silica Gel G (Merck AG, Darmstadt, Germany) containing 2,7,-dichlorofluorescein. Twenty-five grams of Silica Gel G were added to a solution of 30 ml dichlorofluorescein reagent and 20 ml distilled water. Thin-layer plates of about 250 μ in thickness were prepared and dried for at least 2 hr at 110° C. The plates were stored at this temperature until needed.

The 1.0 ml aliquot containing free and esterified cholesterol was evaporated to about 0.1 ml in a vacuum desiccator equipped with a 40° C sand bath maintained by a heating mantle. The desiccator was evacuated by a water aspirator. The concentrated samples were quantitatively

transferred to thin-layer plates with three 50 µl portions of chloroform. Care was taken to keep the spots less than 6 mm in diameter. One reagent blank, one standard, and four plasma samples were spotted on each plate.

A solvent system of petroleum ether-diethyl ether-acetic acid 90:10:1 (v/v/v) was used. Development to 150 mm was achieved in 50 min. The plates were removed from the solvent tank and air-dried 5 min. Lipid spots were detected with an ultraviolet lamp and outlined with a pencil. Thin-layer chromatographic reagent blanks for free and esterified cholesterol regions of appropriate Rf and area were also outlined. The reagent blanks showed no fluorescent spots. A microscope slide was used to scrape the Silica Gel into 15 ml glass stoppered conical centrifuge tubes. Four ml of diethylether-n-hexane 5:95 (v/v) was added to each tube. The samples were extracted 2 min and centrifuged 5 min at 1500 rpm. The clear solvent was carefully decanted into a 15 ml centrifuge tube. The extraction was repeated twice with 3 and 2 ml portions of solvent. The combined extracts were evaporated just to dryness with a gentle stream of nitrogen. The aliquots for total cholesterol were also evaporated to dryness at this point.

FLUOROMETRY

A Turner Model 110 fluorometer (G. K. Turner Associates, Palo Alto, California) was equipped with an ultraviolet lamp (General Electric #F4T4/BL) and a Model 110-655 door. Primary filters numbers 1-60 and 58 (546 mµ excitation) and secondary filter number 23A (570 mµ) were used. The fluorescence was measured at 10× sensitivity in 12 × 75 mm Pyrex tubes.

The cholesterol fluorescence was developed as described in the Turner Manual of Fluorometric Clinical Procedures (7). A blank tube and 3 aliquots of the standard mixture served as instrument blank and reference standards, respectively. All blanks, standards, and unknowns were treated in the following manner:

1. Pipet 0.2 ml 3:2 acetic acid-chloroform (v/v) and mix.
2. Add 5.0 ml 10:3 chloroform-acetic anhydride (v/v) and mix.
3. Add 0.2 ml concentrated sulfuric acid and mix immediately.

The fluorescence was read between 35-50 min after the addition of sulfuric acid. The fluorometer was zeroed with the instrument reagent blank tube. Repipets (automatic dispensing pipets, Labindustries, Berkeley, California) were used in steps 2 and 3. A Vortex Jr. mixer (Scientific Industries, Incorporated, Queens Village, New York) was used for rapid mixing.

The dichlorofluorecein dye was not extracted by the ether-hexane mixture and did not interfere with the fluorescent measurement. Typical readings for the reagent blanks for the free and esterified cholesterol areas were 1.0 and 2.5% of full scale, respectively. Rat plasma with a free to esterified ratio of 36:64 gave readings of approximately 38 to 68% full scale, respectively.

RESULTS AND DISCUSSION

Recovery experiments were performed on free, esterified, and mixed standards. Equal amounts of standards were micropipetted on thin-layer plates and in tubes. The thin-layer chromatographic recoveries are expressed as percent of the direct tube readings. Table I shows the results of the recovery experiments. The recovery of rat plasma samples

based on direct total cholesterol determinations is also included. The separate free and esterified cholesterol standards were run on the same plate. The standard errors for the direct and the thin-layer separated standards were the same.

A cholesterol and cholesterol palmitate mixed standard was analyzed repeatedly over a period of two months. Table II shows the accuracy of determining the percent composition of the mixed standard based on 11 determinations.

The average plasma free cholesterol of six female Sprague-Dawley rats which were fasted 24 hr was found to be 36.6 ± 0.4% of the total cholesterol.

The results presented indicate that free and esterified cholesterol separated by thin-layer chromatography can be quantitatively determined by fluorometric means. The speed of separation and the relatively simple operations employed are distinct advantages over tedious column chromatographic or precipitation methods previously described. The method was also found to be satisfactory for determining free and esterified cholesterol from chloroform-methanol extracts of liver and adrenal glands.

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TABLE 1 THIN-LAYER CHROMATOGRAPHIC RECOVERY EXPERIMENTS

Sample	Number of Samples	% Recovery
Cholesterol	6	96.1 ± 0.2
Cholesterol palmitate	6	97.5 ± 0.3
Mixed standard*	11	95.0 ± 1.5
Rat plasma*	6	96.0 ± 1.3

*Values represent the sum of the free and esterified cholesterol compared to direct total cholesterol readings.

TABLE 2 THIN-LAYER CHROMATOGRAPHIC ACCURACY OF DETERMINING THE PERCENT COMPOSITION OF A STANDARD CONSISTING OF A MIXTURE OF FREE AND ESTERIFIED CHOLESTEROL

	Calculated*	Average of 11 Determinations	Mean Relative Error
	%	%	%
Cholesterol	28.4	28.9 ± 0.2	+1.8
Cholesterol palmitate	71.6	71.1 ± 0.2	-0.7

*The percent composition was based on the weights of standards measured on a microbalance.

Rapid method for determining free and esterified cholesterol in plasma extracts

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SUMMARY A method has been developed for the quantitative measurement of free and esterified cholesterol in extracts

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TABLE 1 MEAN RECOVERY OF FREE AND ESTERIFIED CHOLESTEROL STANDARDS AFTER TLC

Sample	Number of Determinations	Known*	Found ± SEM	Recovery
		μg	μg	%
Mixture	11			
Cholesterol and cholesteryl palmitate		8.97†	8.69 ± 0.1	96.9
		22.6‡	21.4 ± 0.4	94.7
Cholesterol	6	38.4	37.0 ± 0.2	96.2
Cholesteryl palmitate	6	40.0	39.0 ± 0.1	97.5

* The known values for cholesterol were based on the weights of standards on a microbalance.

† Free cholesterol.

‡ Esterified cholesterol.

measured at 10× sensitivity in 12 × 75 mm Pyrex tubes.

The cholesterol fluorescence was developed as described in the Turner Manual of Fluorometric Clinical Procedures (7). A blank tube and three aliquots of the standard mixture served as instrument blank and reference standards, respectively. All blanks, standards, and unknowns were treated in the following manner:

(*a*) Pipette 0.2 ml of acetic acid–chloroform 3:2 (v/v) and mix.

(*b*) Add 5.0 ml of chloroform–acetic anhydride 10:3 (v/v) and mix.

(*c*) Add 0.2 ml of concentrated sulfuric acid and mix immediately.

The fluorescence was read between 35 and 50 min after the addition of sulfuric acid. The fluorometer was zeroed with the instrument reagent blank tube. "Repipets" (automatic dispensing pipettes, Labindustries, Berkeley, Calif.) were used in steps (*b*) and (*c*). A Vortex Junior mixer (Scientific Industries, Inc., Queens Village, New York) was used for rapid mixing.

The dichlorofluorescein dye was not extracted by the ether–hexane mixture and did not interfere with the fluorescence measurement. Typical readings for the reagent blanks for the free and esterified cholesterol areas were 1.0 and 2.5% of full scale, respectively. Forty micrograms of cholesterol gave a reading of approximately 79% of full scale under the operating conditions described.

Results and Discussion. Recovery experiments were performed on replicates of free, esterified, and mixed standards. The recovery of standards from thin-layer plates was found to be >95% (Table 1).

Extracts of six different rat plasma samples were analyzed directly for total cholesterol and, after thin-layer separation, for free and esterified cholesterol. The sum of the free and esterified cholesterol values was compared with that of the total cholesterol to determine the thin-layer chromatographic recovery; the mean recovery was found to be 96.0%. The method was also found to be satisfactory for determining free and esterified cholesterol from chloroform extracts of liver and adrenal glands.

The mean percentage of plasma cholesterol that was unesterified was determined on extracts from six female Sprague-Dawley rats which had been fasted 24 hr. It was found to be 36.6 ± 0.4% of the total cholesterol.

The results presented indicate that free and esterified cholesterol separated by TLC can be quantitiatively determined by fluorometric means. The speed of separation and the relative simplicity of the operations employed are distinct advantages over tedious column chromatographic or precipitation methods previously described.

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